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Mechanism for Clastogenic Activity of Naphthalene

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September 29, 2015

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14. ABSTRACT

Naphthalene incubations form DNA adducts in vitro in a dose dependent manner in both mouse and rat tissues. Rodent tissue incubations with naphthalene indicate that naphthalene forms as many DNA adducts as Benzo(a)pyrene, a known DNA binding carcinogen. The mouse airway has the greatest number of DNA adducts, corresponding to the higher metabolic activation of naphthalene in this location. Both rat tissues, the rat olfactory (tumor target) and the airways (non-tumor target), have similar levels of NA-DNA adducts, indicating that short term measures of initial adduct formation do not directly correlate with sites of tumor formation in the NTP bioassays.

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1. Introduction

There are two primary sources of naphthalene (NA) exposure to military personnel and to their families: the use of JP-8 as a common fuel for both aircraft and transport vehicles and mainstream and sidestream tobacco smoke. Jet fuels contain 1-3% naphthalene by weight, DoD uses an estimated 5.5 billion gallons of JP-8 annually. Naphthalene is the single most prevalent aromatic hydrocarbon in second hand smoke exceeding the levels of polycylic aromatic hydrocarbons like benzo(a)pyrene (BaP) nearly 250 fold (Witschi et al, Carcinogenesis 18, 2035, 1997). The EPA has listed NA as a probable human carcinogen based on the results of the cancer bioassay conducted in both mice and rats. However, there are many groups arguing that NA-induced respiratory tumors in rodents are not relevant to estimating the risks to exposed human populations (see Piccirillo et al, Regul Toxicol Pharmacol. 62: 433, 2012) based on the much slower rates of microsomal NA metabolism in primate compared to rodent respiratory tissue, on the finding that NA is not mutagenic in most *in vitro* short term assays and on epidemiologic data showing that nasal tumors in humans are very rare. This work is intended to provide solid evidence for or against a clastogenic mechanism of action for NA in rodent and non-human primate respiratory tissues. Determining the ability of fresh non-human primate tissues to generate NA metabolites that bind to DNA using ultrasensitive and highly specific assays would provide a mechanistic basis for assessing the risks of NA exposure. If these studies demonstrate binding of metabolites generated in primate tissues to DNA, either removal of NA from fuel sources or additional engineering controls could be established to further protect personnel.

2. Keywords

naphthalene, DNA adducts, clastogen, metabolite

3. Overall Project Summary

All IACUC and ACURO approvals were obtained quickly at the start of project and the subcontract between LLNL and UC Davis (UCD) was executed at the start of December 2014. During the reporting period all the rodent exposures were completed. Rhesus monkey exposures on animals culled from the colony at the UCD California National Primate Research Center (CNPRC) need to be conducted during the fall of 2015.

As proposed, all *in vitro* exposures were conducted using fresh micro-dissected tissues obtained at UCD as described in Van Winkle et al (1996). Tissues were incubated with 2.5, 25, or 250 μM ^{14}C-NA, 25 or 50 μM ^{14}C-benzo(a)pyrene (BaP), or unlabeled sham controls. The groups were treated with NA at 250 and 2.5 μM to test the upper and lower limits of exposure based on John Morris' published (2012) calculations of an upper limit for delivered dose at 250 μM as equivalent to the 10 ppm OSHA exposure limit for NA. Tissues were incubated for 60 minutes followed by 12-15 rinses with ethanol to remove unbound NA or BaP. Tissues were rinsed until the rinse no longer had excess ^{14}C. DNA was isolated from tissues with Qiagen DNeasy kits according to the manufacturer protocols with modification. Two proteinase K digestions were done to assure complete removal of protein. DNA purity was assessed using UV absorbance at 260/280 nm. DNA samples were then prepared for graphitic carbon ^{14}C-AMS analyses using standard procedures (Ognibene et al, 2003; Ognibene et al, 2015a). The NA-DNA adduct levels are depicted in Figures 1-4.

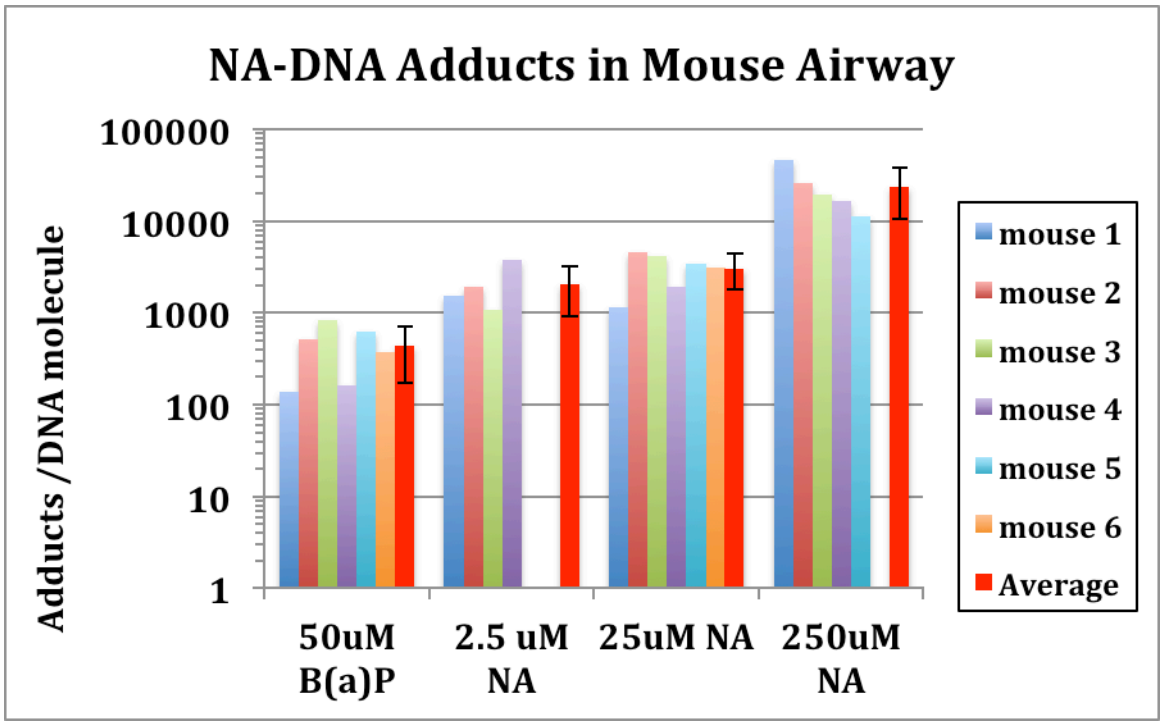

Figure 1. Adducts of BaP or NA per genome in mouse airway. Six replicates were prepared for each dose group with one sample failure at the 250 μM NA and two sample failures at the 2.5 μM NA dose. The average is shown for each dose group.

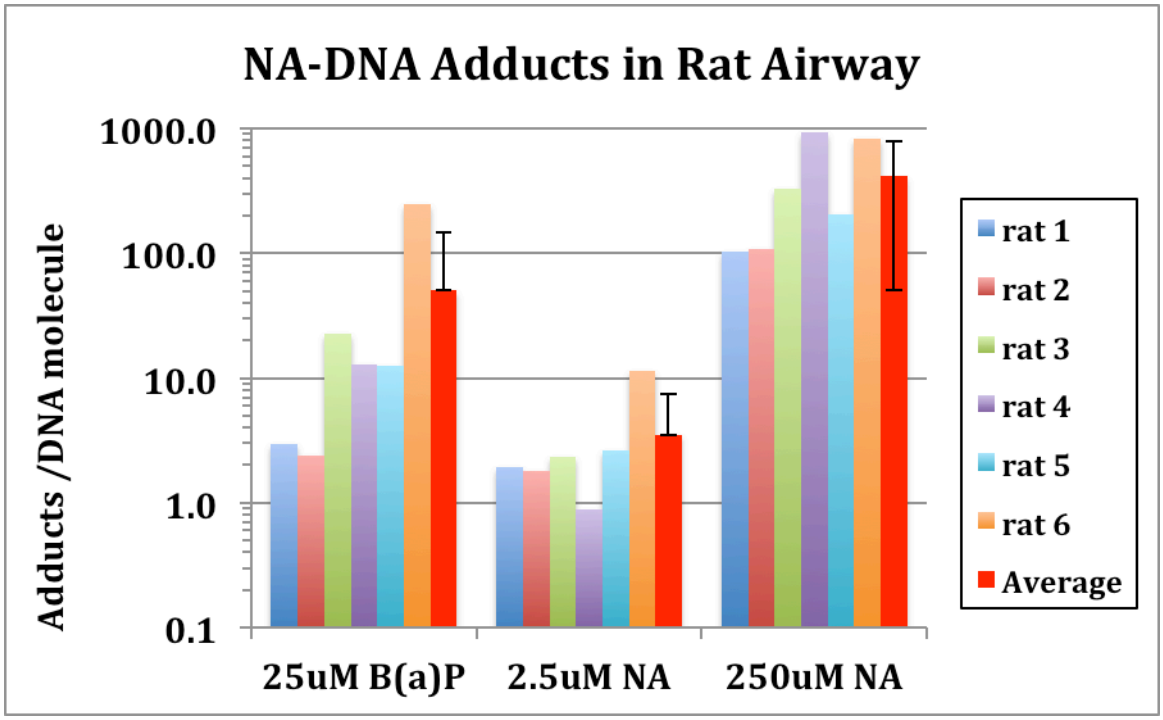

Figure 2. Adducts of BaP and NA per genome in rat airway. Six replicates were prepared and the average is shown for each dose group.

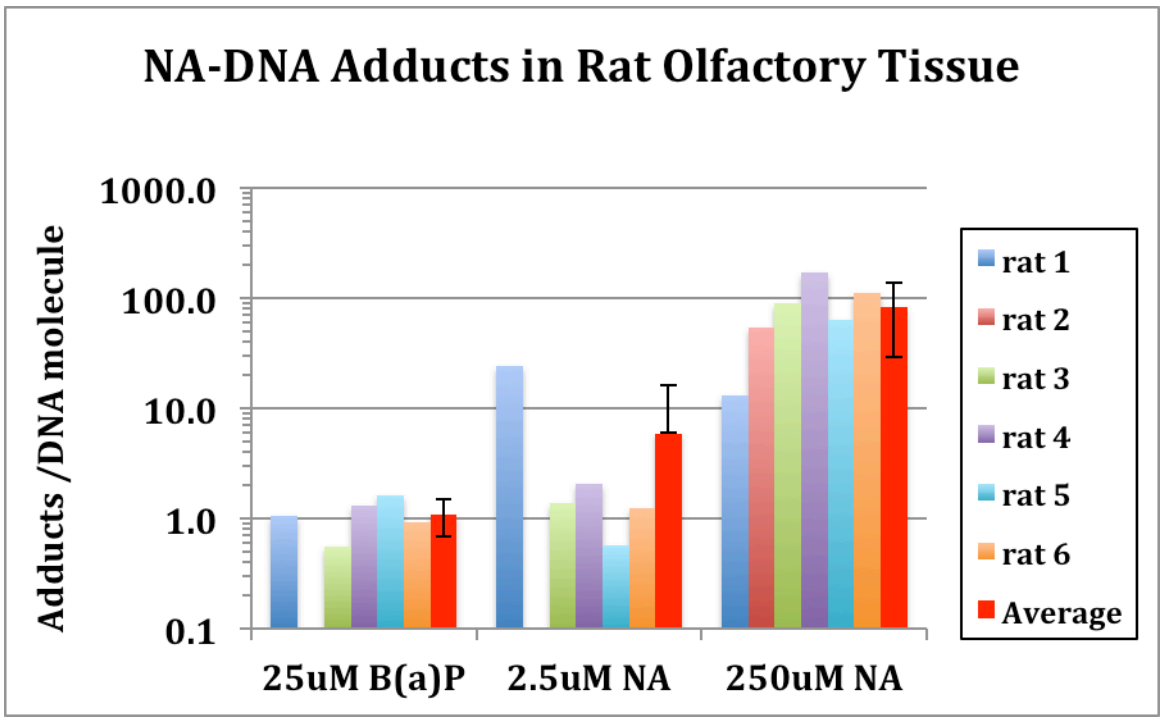

Figure 3. Adducts of BaP and NA per genome in rat olfactory tissue. Six replicates were prepared for each dose group with one sample failure at each the 25 μM BaP and the 2.5 μM NA dose. The average is shown for each dose group.

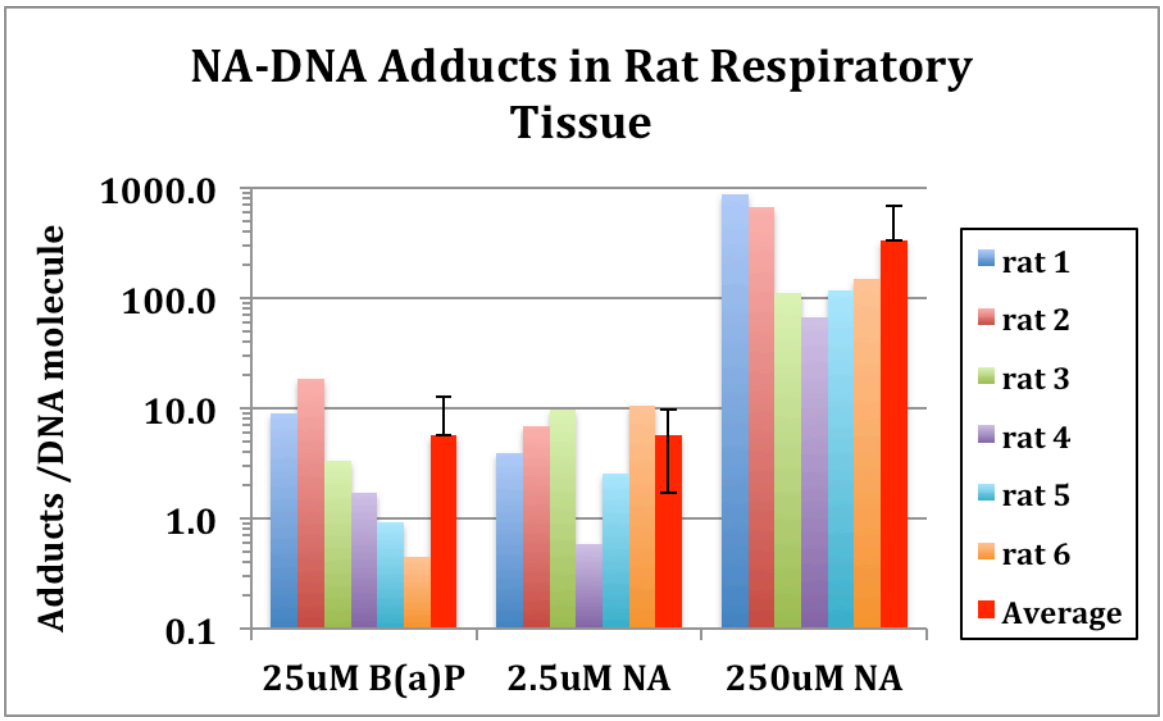

Figure 4. Adducts of BaP and NA per genome in rat respiratory tissue. Six replicates were prepared and the average is shown for each dose group.

Based on the National Toxicology Program (NTP) carcinogenesis bioassays (North et al, 2008), we expected to see the highest level of NA-DNA adducts correspond to the recorded cancer sites following NA exposures: bronchiolar alveolar carcinomas in the airways of female mice and neuroblastomas in the nasal epithelium of rats. Instead, we found the highest measured frequency of NA-DNA adducts in mouse airway, followed by rat airway. Rat olfactory tissue had low levels of adducts similar to rat respiratory tissue. Rat airway did not develop tumors in the NTP bioassay (North et al, 2008). Our experiments did not examine adduct stability, only whether adducts formed upon exposure.

The experiments aimed at determining which DNA bases were adducted by specific NA metabolites have been more difficult to complete than anticipated. Producing adducted nucleosides of NA metabolites has been slowed by the inability to obtain all the reactive NA metabolites. Our original plan was to perform *in vitro* exposures and purify DNA as described above, followed by DNA digestion and HPLC separation of adducted bases followed by AMS analyses of collected fractions (Buchholz et al, 1999). Adducted based would be identified by co-chromatography with synthetic standards. During the summer of 2015 LLNL acquired a Waters Xevo G2 XS QTOF instrument for qualitative and quantitative sample analysis that has been installed with the new liquid sample AMS interface ((Ognibene et al, 2015b). The QTOF enables accurate mass analyses for a variety of analytical applications, including metabolite profiling, identification, characterization, and quantification of both small and macromolecules. The QTOF is coupled to a Waters Acquity H Class HPLC system used to separate samples for introduction to the liquid sample AMS interface. A flow splitter is configured such that eluent from the HPLC flows to the QTOF for qualitative and quantitative analysis, and to the AMS instrument for isotope ratio measurement. Mass spectra are aligned based on retention times to match AMS results to mass spectrometric analytes. Coupling QTOF and AMS measurements in this way constitutes a powerful improvement to our analytical capabilities since molecular ion mass will identify the analyte and AMS will quantify the adduct level. We anticipate completion of the metabolite-adduct experiments by winter.

4. Key Research Accomplishments

The key research accomplishments to date from the quantitation of NA-DNA adducts in mouse and rat tissues are the following:

- Naphthalene incubations form DNA adducts in a dose dependent manner in both mouse and rat tissues
- Rodent tissue incubations with naphthalene indicate that naphthalene forms as many DNA adducts as Benzo(a)pyrene, a known DNA binding carcinogen, acutely.
- The mouse airway has the greatest number of DNA adducts, corresponding to the higher metabolic activation of naphthalene in this location.
- Both rat tissues, the rat olfactory (tumor target) and the airways (non-tumor target), have similar levels of NA-DNA adducts, indicating that short term measures of initial adduct formation do not directly correlate with sites of tumor formation in the NTP bioassays.

5. Conclusion

The NTP bioassays following NA exposure produced neuroblastomas in the nasal epithelium of rats while rat airways did not grow tumors. If the number of initial NA-DNA adducts was the single key event in determining tumor formation at these sites we would expect more adducts in the rat olfactory epithelium than in the rat airway. However, our data indicates slightly higher formation of adducts in rat airway than rat olfactory tissues. Our experiments quantified NA-DNA adduct formation, but did not assess adduct stability. The lack of tumors in the rat airway of the NTP bioassays suggests the adducts we measured are not stable in the long term and are cleared shortly after formation. Furthermore, the high level of NA-DNA adducts we measured in mouse airway were 60-300x that measured in rat tissues at the 250 μM exposure. Yet, the NTP bioassays found rat nasal tumors more prevalent that mouse airway tumors. It appears as though the airway tissues clear a significant amount of NA-DNA adducts *in vivo*. The rhesus monkey tissue exposures planned for fall 2015 will hopefully help put the rodent studies in context. Also the planned adduct-nucleoside separations may shed some light on adduct stability. It is likely that some of the adducts will not be stable following DNA digestion and nucleoside separation.

6. Publications, Abstracts, & Presentations

An abstract is being submitted to the Society of Toxicology Meeting for spring 2016.

7. Inventions, Patents, & Licenses

None.

8. Reportable Outcomes

None at this time.

9. Other Achievements

None.

10. References

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11. Appendices

Quad chart attached.

Mechanism for Clastogenic Activity of Naphthalene

LC130820
MIPR:10567486

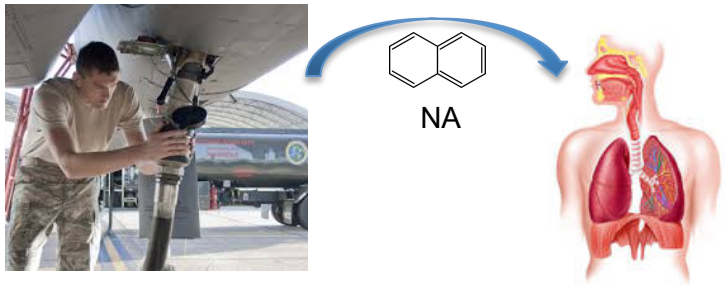
PI: Bruce Buchholz **Org:** Lawrence Livermore National Laboratory **Award Amount:** $165K

Study/Product Aim(s)

- Identify the types of adducts naphthalene (NA) forms with DNA
- Determine whether adduct formation correlates with site selective tumor formation in defined subcompartments of the respiratory tract

Approach

The location of lung and nasal tumors in rodents exposed to NA are highly specific to defined regions within the respiratory tract. We will utilize microdissection methods to isolate live tissue from these target areas. This approach combined with accelerator mass spectrometry (AMS) will determine whether DNA adducts are formed in the target tissue following incubation with ^{14}C-NA.



Determine if naphthalene (NA) in jet fuel and cigarette smoke forms DNA adducts that can lead to cancer in respiratory tissues

Accomplishment: Rodent in vitro exposures complete. Acquisition NA metabolites and generation of adducted DNA bases for HPLC underway.

Timeline and Cost

Activities CY	14	15
Animal Protocol & Contract Complete		
In Vitro Studies for Aim 1 Complete		
In Vitro Studies for Aim 2 Complete		
Sample Analyses Complete		
Data Analyses and Reporting		
Estimated Budget ($K)	**$15**	**$150**

Updated: (15-September-2015)

Goals/Milestones

Q1 Goals – Approval of Animal Protocol and Subcontract Executed
☑ Animal protocols approved
☑ Subcontract with UCD executed
Q2 Goal – Begin Experimental Work
☑ Begin in vitro studies and sample analyses for aims 1& 2
Q3 Goal – Complete Experimental Work
☐ Finish all in vitro studies and sample analyses
Q4 Goals – Complete Data Analyses and Reporting
☐ Complete and submit peer-reviewed publication
☐ Complete and submit final report

Comments/Challenges/Issues/Concerns

- Monkey in vitro exposures to be completed fall 2015.
- Delay in securing metabolite adducted bases has HPLC work for Goal 1 behind schedule.

Budget Expenditure to Date

Projected Expenditure: $165K
Actual Expenditure: $100K